Attorney Docket Number 51590.62078

TITLE OF THE INVENTION

IDENTIFICATION AND CHARACTERIZATION OF MULTIPLE SPLICE
VARIANTS OF THE MU OPIOID RECEPTOR GENE

5   RELATED APPLICATIONS/PATENTS & INCORPORATION BY REFERENCE

This application claims priority to U.S. Provisional Application Serial No.
60/544,534, filed on February 13, 2004 as Attorney Docket No. 830002-2013, the
contents of which are incorporated herein by reference.

Each of the applications and patents cited in this text, as well as each

10   document or reference cited in each of the applications and patents (including during
the prosecution of each issued patent; "application cited documents"), and each of
the PCT and foreign applications or patents corresponding to and/or claiming
priority from any of these applications and patents, and each of the documents cited
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15   incorporated herein by reference. More generally, documents or references are cited
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document or reference cited in each of the herein-cited references (including any
manufacturer's specifications, instructions, etc.), is hereby expressly incorporated

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STATEMENT OF RIGHTS TO INVENTION MADE UNDER FEDERALLY
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TECHNICAL FIELD

The present invention relates to mu-opioid receptor-1 (MOR-1) splice variant
polypeptides, to DNA sequences encoding the splice variants, to DNA sequences

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encompassing non-coding region splice variants, to methods of screening compositions for agonists and antagonists of the splice variant receptor activities and to methods of measuring splice variant binding activities.

5 BACKGROUND ART

Opiates are drugs derived from opium and include morphine, codeine and a wide variety of semisynthetic opioid congeners derived from them and from thebaine, another component of opium. Opioids include the opiates and all agonists and antagonists with morphine-like activity and naturally occurring endogenous and 10 synthetic opioid peptides. Morphine and other morphine-like opioid agonists are commonly used pharmaceutically to produce analgesia.

There are now many compounds with pharmacological properties similar to those produced by morphine, but none has proven to be clinically superior in relieving pain. References to morphine herein will be understood to include 15 morphine-like agonists as well. The effects of morphine on human beings are relatively diverse and include analgesia, drowsiness, changes in mood, respiratory depression, decreased gastrointestinal motility, nausea, vomiting, and alterations of the endocrine and autonomic nervous systems. Pasternak (1993) Clin. Neuropharmacol. 16:1. Doses of morphine need to be tailored based on individual 20 sensitivity to the drug and the pain-sparing needs of the individual. For instance, the typical initial dose of morphine (10mg/70kg) relieves post-operative pain satisfactorily in only two-thirds of patients. Likewise, responses of an individual patient may vary dramatically with different morphine-like drugs and patients may have side effects with one such drug and not another. For example, it is known that 25 some patients who are unable to tolerate morphine may have no problems with an equianalgesic dose of methadone. The mechanisms underlying variations in individual responses to morphine and morphine-like agonists have not been defined.

The analgesic effects of morphine are transduced through opioid receptors in the central nervous system (CNS), located at both spinal and multiple supraspinal 30 sites. Morphine and other agonists induce profound analgesia when administered intrathecally or instilled locally into the dorsal horn of the spinal cord. Several

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mechanisms of action are believed to mediate the inhibition of nociceptive reflexes from reaching higher centers of the brain, including the inhibition of neurotransmitter release by opioid receptors on the termini of primary afferent nerves and post synaptic inhibitory actions on interneurons and on the out-put

5 neurons of the spinothalamic tract.

Profound analgesia can also be produced by the instillation of morphine into the third ventricle or within various sites in the midbrain and medulla, most notably the periaqueductal gray matter, the nucleus raphe magnus, and the locus ceruleus. Although the neuronal circuitry responsible has not been defined, these actions

10 produce enhanced activity in the descending aminergic bulbospinal pathways that exert inhibitory effects on the processing of nociceptive information in the spinal cord. Simultaneous administration of morphine at both spinal and supraspinal sites results in a synergized analgesic response, with a ten-fold reduction in the total dose of morphine necessary to produce equivalent analgesia at either site alone.

15 Morphine also exerts effects on the neuroendocrine system. Morphine acts in the hypothalamus to inhibit the release of gonadotropin releasing hormone (GnRH) and corticotropin-releasing factor (CRF), thus decreasing circulating concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), and adrenocorticotropin (ACTH), and β-endorphin. As a result of the decreased

20 concentrations of pituitary trophic hormones, the concentrations of testosterone and cortisol in the plasma decline. The administration of opiates increases the concentration of prolactin (PRL) in plasma, most likely by reducing the dopaminergic inhibition of PRL secretion. With chronic administration, tolerance eventually develops to the effects of morphine on hypothalamic releasing factors.

25 Opiates can interfere with normal gastrointestinal functioning. Morphine decreases both gastric motility and the secretion of hydrochloric acid in the stomach. Morphine may delay passage of gastric contents through the duodenum for as long as 12 hours. Morphine also decreases biliary, pancreatic and intestinal secretions and delays the digestion of food in the small intestine. Propulsive peristaltic waves

30 in the colon are diminished or abolished after administration of morphine and commonly, constipation occurs. For a detailed review of the physiological effects of

morphine, see Reisine and Pasternak (1996) Goodman & Gilman's The pharmacological basis of therapeutics, Ninth Edition (Hardman et al. eds.) McGraw-Hill pp. 521-555.

5   Morphine also exerts effects on the immune system. The most firmly established effect of morphine is its ability to inhibit the formation of rosettes by human lymphocytes. The administration of morphine to animals causes suppression of the cytotoxic activity of natural killer cells and enhances the growth of implanted tumors. These effects appear to be mediated by actions within the CNS. By contrast, β-endorphin enhances the cytotoxic activity of human monocytes in vitro

10   and increases the recruitment of precursor cells into the killer cell population; this peptide also can exert a potent chemotactic effect on these cells. A novel type of receptor (designated ∈) may be involved. These effects, combined with the synthesis of Proopiomelanocortin (POMC) and preproenkephalin by various cells of the immune system, have stimulated studies of the potential role of opioids in the

15   regulation of immune function. Sibinga and Goldstein (1988) Annu. Rev. Immunol. 6:219.

       Side effects resulting from the use of morphine range from mild to life threatening. Morphine causes constriction of the pupil by an excitatory action on the parasympathetic nerve innervating the pupil. Morphine depresses the cough reflex

20   through inhibitory effects on the cough centers in the medulla. Nausea and vomiting occur in some individuals through direct stimulation of the chemoreceptor trigger zone for emesis, in the postrema of the medulla. Therapeutic doses of morphine also result in peripheral vasodilatation, reduced peripheral resistance and an inhibition of baroreceptor reflexes in the cardiovascular system. Additionally, morphine

25   provokes the release of histamines, which can cause hypotension. Morphine depresses respiration, at least in part by direct effects on the brainstem regulatory systems. In humans, death from morphine poisoning is nearly always due to respiratory arrest. Opioid antagonists can produce a dramatic reversal of severe respiratory depression and naloxone is currently the treatment of choice. High doses

30   of morphine and related opioids can produce convulsions that are not always relieved by naloxone.

The development of tolerance and physical dependence with repeated use is a characteristic feature of all opiates. Dependence seems to be closely related to tolerance, since treatments that block tolerance to morphine also block dependence. In vivo studies in animal models demonstrate the importance of neurotransmitters

5 and their interactions with opioid pathways in the development of tolerance to morphine. Blockade of glutamate actions by noncompetitive and competitive NMDA (N-methyl-D-aspartate) antagonists blocks morphine tolerance. Trujillo and Akil (1991) Science 251:85; and Elliott et al. (1994) Pain 56:69. Blockade of the glycine regulatory site on NMDA receptors has similar effects to block tolerance.

10 Kolesnikov et al. (1994) Life Sci. 55:1393. Administering inhibitors of nitric oxide synthase in morphine-tolerant animals reverses tolerance, despite continued opioid administration. Kolesnikov et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:5162. These studies indicate several important aspects of tolerance and dependence. First, the selective actions of drugs on tolerance and dependence demonstrate that

15 analgesia can be dissociated from these two unwanted actions. Second, the reversal of preexisting tolerance by NMDA antagonists and nitric oxide synthase inhibitors indicates that tolerance is a balance between activation of processes and reversal of those processes. These observations suggest that, by use of selective agonists and/or antagonists, tolerance and dependence in the clinical management of pain can be

20 minimized or disassociated from the therapeutic effects.

In addition to morphine, there are a variety of opioids suitable for clinical use. These include, but are not limited to, Levorphanol, Meperidine, Fentanyl, Methadone, Codeine, Propoxyphene and various opioid peptides. Certain opioids are mixed agonists/antagonists and partial agonists. These include pentazocine,

25 nalbuphine, butorphanol, and buprenorphine. The pharmacological effects of levorphanol closely parallel those of morphine although clinical reports suggest that levorphanol produces less nausea.

Meperidine exerts its chief pharmacological effects on the central nervous system and the neural elements in the bowel. Meperidine produces a pattern of

30 effects similar but not identical to those described for morphine. In equianalgesic doses, meperidine produces as much sedation, respiratory depression, and euphoria

as morphine. The pattern of unwanted side effects that follow the use of meperidine are similar to those observed after equianalgesic doses of morphine, except that constipation and urinary retention are less common.

5   Fentanyl is a synthetic opioid estimated to be 80 times as potent as morphine as an analgesic. High doses of fentanyl can result in severe toxicity and produce side effects including muscular rigidity and respiratory depression.

Methadone is an opioid with pharmacological properties similar to morphine. The properties of methadone include effective analgesic activity, efficacy by the oral route and persistent effects with repeated administration. Side effects include

10   detection of miotic and respiratory-depressant effects for more than 24 hours after a single dose, and marked sedation is seen in some patients. Effects on cough, bowel motility, biliary tone and the secretion of pituitary hormones are qualitatively similar to those of morphine. In contrast to morphine, codeine is approximately 60% as effective orally as parenterally, both as an analgesic and as a respiratory depressant.

15   Codeine has an exceptionally low affinity for opioid receptors, and the analgesic effect of codeine is due to its conversion to morphine. However, codeine's antitussive actions probably involve distinct receptors that bind codeine specifically.

Propoxyphene produces analgesia and other CNS effects that are similar to those seen with morphine. It is likely that at equianalgesic doses the incidence of

20   side effects such as nausea, anorexia, constipation, abdominal pain, and drowsiness would be similar to those of codeine.

Opioid antagonists have therapeutic utility in the treatment of overdosage with opioids. As understanding of the role of endogenous opioid systems in pathophysiological states increases, additional therapeutic indications for these

25   antagonists will emerge. If endogenous opioid systems have not been activated, the pharmacological actions of opioid antagonists depend on whether or not an opioid agonist has been administered previously, the pharmacological profile of that opioid and the degree to which physical dependence on an opioid has developed. The antagonist naloxone produces no discernible subjective effects aside from slight

30   drowsiness. Naltrexone functions similarly, but with higher oral efficacy and a longer duration of action. Currently, naloxone and naltrexone are used clinically to

treat opioid overdoses. Their potential utility in the treatment of shock, stroke, spinal cord and brain trauma, and other disorders that may involve mobilization of endogenous opioids remains to be established.

The complex interactions of morphine and drugs with mixed
5 agonist/antagonist properties are mediated by multiple classes of opioid receptors. Opioid receptors comprise a family of cell surface proteins, which control a range of biological responses, including pain perception, modulation of affective behavior and motor control, autonomic nervous system regulation and neuroendocrinological function. There are three major classes of opioid receptors in the CNS, designated
10 mu, kappa and delta, which differ in their affinity for various opioid ligands and in their cellular distribution. The different classes of opioid receptors are believed to serve different physiologic functions. Olson et al. (1989) Peptides 10:1253; Lutz and Pfister (1992) J. Receptor Res. 12:267; and Simon (1991) Medicinal Res. Rev. 11:357. Morphine produces analgesia primarily through the mu-opioid receptor.
15 However, among the opioid receptors, there is substantial overlap of function as well as of cellular distribution.

The mu-opioid receptor mediates the actions of morphine and morphine-like opioids, including most clinical analgesics. In addition to morphine, several highly selective agonists have been developed for mu-opioid receptors, including [D-
20 Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO), levorphanol and methadone. Differential sensitivity to antagonists, such as naloxonazine, indicates the pharmacological distinctions between the mu-opioid receptor subtypes, mu$_1$ and mu$_2$. Several of the opioid peptides will also interact with mu-opioid receptors.

There are three distinct families of endogenous opioid peptides, the
25 enkephalins, endorphins and dynorphins, where each peptide is derived from a distinct precursor polypeptide. Mu-opioid receptors have a high affinity for the enkephalins as well as β-endorphin and dynorphin A. For review, see Reisine and Pasternak (1996).

Members of each known class of opioid receptor have been cloned from
30 human cDNA and their predicted amino acid sequences have been determined. Yasuda et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6736; and Chen et al. (1993)

Mol. Pharmacol. 44:8. The opioid receptors belong to a class of transmembrane spanning receptors known as G-protein coupled receptors. G-proteins consist of three tightly associated subunits, alpha, beta and gamma (1:1:1) in order of decreasing mass. Following agonist binding to the receptor, a conformational

5   change is transmitted to the G-protein, which causes the G-alpha subunit to exchange a bound GDP for GTP and to dissociate from the beta and gamma subunits. The GTP-bound form of the alpha subunit is typically the effector-modulating moiety. Signal amplification results from the ability of a single receptor to activate many G-protein molecules, and from the stimulation by G-alpha-GTP of

10   many catalytic cycles of the effector.

Most opioid receptor-mediated functions appear to be mediated through G-protein interactions. Standifer and Pasternak (1997) Cell Signal. 9:237. Antisense oligodeoxynucleotides directed against various G-protein alpha subunits were shown to differentially block the analgesic actions of the mu-, delta-, and kappa- opioid

15   agonists in mice. Standifer et al. (1996) Mol. Pharmacol. 50:293.

The amino acid sequences of the opioid receptors are approximately 65% identical, and they have little sequence similarity to other G-protein-coupled receptors except for somatostatin. Reisine and Bell (1993) Trends Neurosci. 16:506. The regions of highest similarity in sequence are the sequences predicted to lie in the

20   seven transmembrane-spanning regions and the intracellular loops. Regions of amino acid sequence divergence are the amino and carboxy termini and the second and third extracellular loops.

Each receptor subtype has a characteristic pattern of expression. Mu-opioid receptor mRNA is present in the periaqueductal gray, spinal trigeminal nucleus,

25   cuneate and gracile nuclei, and thalamus regions of the brain involved in pain perception and associated with morphine analgesia. Defts et al. (1994) J. Comp. Neurol. 345:46. It is also present in nuclei involved in control of respiration, consistent with the ability of morphine to depress respiration, and in neurons of the area postrema, where morphine has been shown to cause nausea and induce

30   vomiting. Other consequences of mu-opioid receptor activation include miosis, reduced gastrointestinal motility, and feelings of well-being or euphoria. Pasternak

(1993). The pattern of mu-opioid receptor mRNA expression correlates with the brain centers involved in mediating the biological actions of morphine and mu-selective agonists. Delta-opioid receptor mRNA is found in the dorsal horn of the spinal cord. $Kappa_1$-opioid receptor mRNA is expressed in the hypothalamic

5 regions, which may account for many of the neuroendocrine effects of the kappa selective agonists.

Soon after the mu-opioid receptor MOR-1 was cloned (Chen et al. (1993); and Wang et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:10230), antisense experiments confirmed its involvement with morphine analgesia. Rossi et al. (1994)

10 Life Sci. 54:375; and Rossi et al. (1995) FEBS Lett. 369:192. Antisense oligonucleotides directed against MOR-1 mRNA blocked the analgesic actions of morphine in rats, demonstrating that proper translation of the MOR-1 mRNA was essential for modulating morphine analgesia. Antisense approaches have also demonstrated a relationship between MOR-1 activity and ingestive responses.

15 Administration of antisense oligonucleotides directed against MOR-1 mRNA significantly reduced food and water intake and subsequently, body weight in rats.

In recent years, a number of mu-opioid receptor subtypes have been proposed. The first suggestion of mu_1 and mu_2 receptor subtypes came from a combination of binding and pharmacological studies based on the antagonists

20 naloxonazine and naloxazone. Wolozin and Pasternak (1981) Proc. Natl. Acad. Sci. U.S.A. 78:6181; Reisine and Pasternak (1996); and Pasternak (1993). A gene encoding a mu receptor, MOR-1, has been identified. Min et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91:9081; Giros et al. (1995) Life Sci. 56:PL369; and Liang et al. (1995) Brain Res. 679:82. The MOR-1 cDNA consists of exons 1-4, which total

25 1610 bp in length and encode 398 amino acids. More recently, pharmacological and molecular differences between morphine and morphine-6β-glucuronide (M6G) have suggested yet another mu-opioid receptor subtype. Pasternak and Standifer (1995) Trends Pharmacol. Sci. 16:344; Rossi et al. (1995); and Rossi et al. (1996) Neurosci. Lett. 216:1.

30 Antisense oligonucleotides directed against selected exons within the MOR-1 mRNA revealed interesting therapeutic patterns of morphine and M6G analgesia,

with some MOR-1 exons implicated in the analgesic actions of one drug, but not the other. Rossi et al. (1997) J. Pharmacol. Exp. Ther. 281:109; and Rossi et al. (1995). Although the two analgesics were known to act through different receptors, the sensitivity of the effect of both analgesics to at least six different MOR-1 antisense

5   probes implied that both receptors were closely associated with MOR-1, raising the possibility of pharmacologically relevant MOR-1 splice variants. Pasternak and Standifer (1995); and Rossi et al. (1995). Alternative splicing has been observed with a number of G-protein-coupled receptors, including somatostatin 2 (Vanetti et al. (1998) FEBS Lett. 311:290), dopamine D2 (Guiramand et al. (1995) J. Biol.

10   Chem. 270:7354), prostaglandin EP3 (Namba et al. (1993) Trends Pharmacol. Sci. 16:246), serotonin receptor subtypes 5-HT$_4$ and 5-HT$_7$ (Lucas and Hen. (1995) Trends Pharmacol. Sci. 16:246) and MOR-1 (Bare et al. (1994) FEBS Lett. 354:213; and Zimprich et al. (1995) FEBS Lett. 359:142).

Several opioid receptor splice variants have been identified and

15   characterized. At least two MOR-1 splice variants are known, the human MOR-1A and the rat MOR-1B. Bare et al. (1994); and Zimprich et al. (1995). The hMOR-1A splice variant consists of exons 1, 2, 3 and a new exon 3a, and was determined to possess ligand binding characteristics similar to the full-length MOR-1. Bare et al. (1994). The rMOR-1B splice variant consists of exons 1, 2, 3 and a new exon 5, and

20   like hMOR-1A, differs from MOR-1 only in length and amino acid composition at the carboxy-terminal tail. Zimprich et al. (1995). MOR-1B has affinity to opioid compounds similar to that of MOR-1, but is much more resistant to agonist-induced desensitization than MOR-1. The C-terminal differences between MOR-1 and MOR-1A or MOR-1B could have effects on receptor coupling or receptor transport

25   and localization. About twenty splice variants of the mouse MOR-1 gene (comprised of nine exons) have been identified and characterized. (see, e.g., PCT/US99/15974, published as WO 00/04046 and PCT/US02/20665, published as WO 03/002718 A2). The MOR-1 splice variants are potential targets for the modulation of physiological effects resulting from mu-opioid receptor activity. In

30   addition, five splice variants of the kappa3-related opioid receptor (i.e., opioid receptor-like receptor (ORL-1), OFQ receptor or nociceptin receptor) have been

identified and characterized, suggesting an analogous system of modulation to that of the mu class of receptors. (PCT/US99/15977, published as WO 00/04151). Splice variants that were under control of two distinct promoters in the mouse *Oprm* gene have been reported (see, e.g., Pan et al. (1999) Molecular Pharmacology 56, 396-403, Pan et al. (2000) FEBS Letters 466, 337-340, Pan et al. (2002) Gene 295, 97-108 and Pan et al. (2001) Proc.Natl.Acad.Sci.U.S.A 98, 14084-14089). Two additional human MOR-1 splice variants, hMOR-1O and hMOR-1X (see, e.g., Pan et al. (2003) Biochem Biophys Res Commun. 301, 1057-1061), have also been identified.

Availability of polynucleotide sequences of opioid receptor splice variants, and, in the case of splice variants in coding regions, the corresponding polypeptide sequences, will significantly increase the capability to design pharmaceutical compositions, such as analgesics, with enhanced specificity of function. In general, the availability of these polynucleotide and polypeptide sequences will enable efficient screening of candidate compositions. The principle in operation through the screening process is straightforward: natural agonists and antagonists bind to cell-surface receptors and channels to produce physiological effects; certain other molecules can produce physiological effects and act as therapeutic pharmaceutical agents. Thus, the ability of candidate drugs to bind to opioid receptor splice variants can function as an extremely effective screening criterion for the selection of pharmaceutical compositions with desired functional efficacy and specificity.

DISCLOSURE OF THE INVENTION

The invention encompasses MOR-1 splice variant polypeptides or polypeptide fragments or homologs thereof retaining MOR-1 activity.

The invention further encompasses a MOR-1 splice variant polynucleotide, encoding MOR-1 splice variant polypeptides or polypeptide fragments or homologs thereof retaining MOR-1 activity, and noncoding mRNA splice variants and complementary strands thereto.

The invention further encompasses a polynucleotide, or a complementary strand thereto that hybridizes under stringent conditions, comprising at least 15

consecutive nucleotides of an MOR-1 splice variant polynucleotide where the polynucleotide contains promoter elements.

The invention further encompasses methods of screening compositions for an opioid activity by obtaining a control cell that does not express a recombinant or

5   endogenous opioid receptor, obtaining a test cell that expresses a recombinant MOR-1 splice variant polypeptide, contacting the control cell and test cell with an amount of an opioid sufficient to exert a physiologic effect, separately measuring the physiologic effect of the composition on the control cell and test cell and comparing the physiologic effect of the composition to the physiologic effect of the opioid,

10   where determination of a physiologic effect of the composition is expressed relative to that of the opioid.

The invention further encompasses methods of screening compositions for an opioid activity by obtaining a control polypeptide that is not a recombinant opioid receptor, obtaining a test polypeptide that is a recombinant MOR-1 splice variant

15   polypeptide, contacting a composition with the control polypeptide and the test polypeptide, contacting the test polypeptide with an amount of an opioid sufficient to measurably bind the test polypeptide, measuring the binding of the composition and the opioid, and comparing the test polypeptide binding of the composition to that of the opioid, where determination of binding of the composition is expressed

20   relative to that of the opioid.

The invention further encompasses methods of screening compositions for differential or selective opioid activity comprising obtaining a first and second test polypeptide that are MOR-1 splice variant polypeptide fragments and contacting each with a composition, measuring the binding affinity of the composition to the

25   first and second test polypeptides and comparing the binding of the composition and the first test polypeptide to that of the second test polypeptide where differential activity is expressed as a ratio of the two binding affinities.

The invention further encompasses methods of screening compositions for a MOR-1 binding protein by obtaining a control cell that does not express a

30   recombinant or endogenous opioid receptor, obtaining a test cell that expresses a recombinant MOR-1 splice variant polypeptide, contacting the control cell extract

and test cell extract with the MOR-1 splice variant polypeptide and comparing the test cell extract binding to that of the control cell extract binding with the MOR-1 splice variant polypeptide of interest to identify a MOR-1 binding protein.

5   The invention further encompasses a method for regulating morphine analgesia in a subject by altering of MOR-1 splice variant polypeptide activity. Activity can be regulated by administering antigen binding fragments, agonists, antagonists, small molecule ligands, antisense nucleic acids or siRNA to a subject in an amount and for a duration sufficient to regulate morphine analgesia. The antigen binding fragment, agonist, antagonist, small molecule ligand, antisense nucleic acid

10   or siRNA is directed to an MOR-1 splice variant polypeptide fragment or a homolog thereof or an MOR-1 splice variant mRNA. Morphine analgesia can also be regulated by homodimerization or homooligomerization among the MOR-1 splice variants and heterodimerization or heterooligomerization between the variants and other opioid or non-opioid receptors.

15   The invention further encompasses regulating opioid activity by administering a DNA plasmid vector containing an MOR-1 splice variant polynucleotide. The DNA plasmid vector thereby expresses an mRNA splice variant that may encode an MOR-1 polypeptide in a subject in an amount of and duration sufficient to regulate morphine analgesia. Activity can also be regulated by

20   administering an antisense nucleic acid or siRNA complementary to an MOR-1 splice variant polynucleotide, thereby blocking gene expression in a subject in an amount and duration sufficient to regulate morphine analgesia. Opioid activity can also be regulated by homodimerization or homooligomerization among the MOR-1 splice variants and heterodimerization or heterooligomerization between the variants

25   and other opioid or non-opioid receptors.

The invention further encompasses antigen-binding fragments specific for the MOR-1 splice variant polypeptides described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

30   FIG. 1A shows the human mu opioid receptor gene structure and alternative splicing. Exons and introns are showed by boxes and horizontal lines, respectively.

FIG. 1B shows the partial cDNA sequences of the variants with translated amino acid sequences. The complete cDNA and deduced amino acid sequences of hMOR-1A, hMOR-1B1, hMOR-1B2, hMOR-1B3, hMOR-1B4, hMOR-1B5, and hMOR-1Y have been deposited in the GenBank database (Accession numbers:

5   AY225404, AY309001, AY309005, AY309006, AY309007, AY309008, and AY309009). The cAMP- and cGMP-dependent protein kinase phosphorylation sites, caseine kinase II phosphorylation sites, tyrosine kinase phosphorylation site and translation stop codon are indicated by #, @, & and * respectively.

FIG. 2 shows a schematic of the protein structure for the human MOR-1

10   variants.

FIG. 3A depicts the nucleotide sequence and amino acid sequence of hMOR-1B1.

FIG. 3B depicts the nucleotide sequence and amino acid sequence of hMOR-1B2.

15   FIG. 3C depicts the nucleotide sequence and amino acid sequence of hMOR-1B3.

FIG. 3D depicts the nucleotide sequence and amino acid sequence of hMOR-1B4.

FIG. 3E depicts the nucleotide sequence and amino acid sequence of hMOR-

20   1B5.

FIG. 3F depicts the nucleotide sequence and amino acid sequence of hMOR-1Y.

FIG. 4 shows an alignment of the human MOR-1 amino acid sequences.

FIG. 5 shows the partial cDNA sequences of the variants with translated

25   amino acid sequences. The stop codons are indicated by *. The complete cDNA and deduced amino acid sequences of rMOR-1A, rMOR-1C1, rMOR-1C2, rMOR-1D, rMOR-1B2 and rMOR-1E have been deposited in the GenBank database (Accession numbers. AY309000, AY225402, AY225403, AY309002, AY309003 and AY309004).

30   FIG. 6A depicts the nucleotide sequence and amino acid sequence of rMOR-1B2.

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FIG. 6B depicts the nucleotide sequence and amino acid sequence of rMOR-1C1.

FIG. 6C depicts the nucleotide sequence and amino acid sequence of rMOR-1C2.

5   FIG. 6D depicts the nucleotide sequence and amino acid sequence of rMOR-1D.

FIG. 6E depicts the nucleotide sequence and amino acid sequence of rMOR-1E.

10   ## BEST MODE FOR CARRYING OUT THE INVENTION

In view of the strong pharmacological evidence for distinct mu-opioid receptors, alternative splicing of the MOR-1 gene has been explored further. It has now been determined that the MOR-1 gene is subject to alternative splicing that produces novel splice variant forms of the mRNA and/or receptor. New exons for
15   the MOR-1 gene have been identified, which combine to yield novel MOR-1 splice variant polynucleotides. These splice variant polynucleotides and the polypeptides encoded thereby are potential targets for modulating morphine analgesia and opioid-mediated ingestive responses.

The invention further encompasses isolated MOR-1 splice variant
20   polynucleotides having the sequences of SEQ ID NOS: 50, 52, 54, 56, 58 or 60. In addition to SEQ ID NOS 50, 52, 54, 56, 58 or 60, the polynucleotide sequences can be any sequence of the appropriate genetic code to encode any of the MOR-1 splice variant polypeptides having the sequence of SEQ ID NOS: 51, 53, 55, 57, 59 or 61. Preferably, the polynucleotide is at least 15 consecutive nucleotides.

25   The invention further encompasses isolated MOR-1 splice variant polynucleotides having the sequences of SEQ ID NOS: 79, 81, 83, 85 or 87. In addition to SEQ ID NOS 79, 81, 83, 85 or 87, the polynucleotide sequences can be any sequence of the appropriate genetic code to encode any of the MOR-1 splice variant polypeptides having the sequence of SEQ ID NOS: 80, 82, 84, 86 or 88.
30   Preferably, the polynucleotide is at least 15 consecutive nucleotides.

A "polynucleotide" is a polymeric form of nucleotides of any length, which contain deoxyribonucleotides, ribonucleotides, and analogs in any combination. Polynucleotides may have three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes double-, single-stranded, and triple-helical molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double stranded form and each of two complementary forms known or predicted to make up the double stranded form of either the DNA, RNA or hybrid molecule.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thiolate, and nucleotide branches. The sequence of nucleotides may be further modified after polymerization, such as by conjugation, with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides or solid support.

An "isolated" polynucleotide or polypeptide is one that is substantially free of the materials with which it is associated in its native environment. By substantially free, is meant at least 50%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90% free of these materials.

The invention further comprises a complementary strand to the MOR-1 splice variant polynucleotide.

The complementary strand can be polymeric and of any length, and can contain deoxyribonucleotides, ribonucleotides, and analogs in any combination.

Hybridization reactions can be performed under conditions of different "stringency." Conditions that increase stringency of a hybridization reaction are

well known. See for examples, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al. 1989). Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37°C, 50°C, and 68°C; buffer concentrations of 10 x SSC, 6 x SSC, 1 x SSC, 0.1 x SSC (where SSC is 0.15

5   M NaCl and 15 mM citrate buffer) and their equivalent using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2 or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 x SSC, 1 x SSC, 0.1 x SSC, or deionized water.

     The invention further encompasses polynucleotides encoding functionally

10   equivalent variants and derivatives of the MOR-1 splice variant polypeptides and functionally equivalent fragments thereof which may enhance, decrease or not significantly affect properties of the polypeptides encoded thereby. These functionally equivalent variants, derivatives, and fragments display the ability to retain MOR-1 activity. For instance, changes in a DNA sequence that do not change

15   the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect properties of the encoded polypeptide. Conservative amino acid substitutions are glycine/alanine; valine/isoleucine/leucine;

20   asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine/methionine; lysine/arginine; and phenylalanine/tyrosine/tryptophan.

     The invention further encompasses the MOR-1 splice variant polynucleotides contained in a vector molecule or an expression vector and operably linked to a promoter element if necessary.

25   A "vector" refers to a recombinant DNA or RNA plasmid or virus that comprises a heterologous polynucleotide to be delivered to a target cell, either in vitro or in vivo. The heterologous polynucleotide may comprise a sequence of interest for purposes of therapy, and may optionally be in the form of an expression cassette. As used herein, a vector need not be capable of replication in the ultimate

30   target cell or subject. The term includes cloning vectors for translation of a polynucleotide encoding sequence. Also included are viral vectors.

The term "recombinant" means a polynucleotide of genomic cDNA, semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in an arrangement not found in nature.

"Heterologous" means derived from a genetically distinct entity from the rest

5 of the entity to which it is being compared. For example, a polynucleotide, may be placed by genetic engineering techniques into a plasmid or vector derived from a different source, and is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence other than the native sequence is a heterologous promoter.

10 The polynucleotides of the invention may comprise additional sequences, such as additional encoding sequences within the same transcription unit, controlling elements such as promoters, ribosome binding sites, polyadenylation sites, additional transcription units under control of the same or a different promoter, sequences that permit cloning, expression, homologous recombination, and

15 transformation of a host cell, and any such construct as may be desirable to provide embodiments of this invention.

A "host cell" denotes a prokaryotic or eukaryotic cell that has been genetically altered, or is capable of being genetically altered by administration of an exogenous polynucleotide, such as a recombinant plasmid or vector. When referring

20 to genetically altered cells, the term refers both to the originally altered cell and to the progeny thereof.

Polynucleotides comprising a desired sequence can be inserted into a suitable cloning or expression vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification. Polynucleotides can be introduced into

25 host cells by any means known in the art. The vectors containing the polynucleotides of interest can be introduced into the host cell by any of a number of appropriate means, including direct uptake, endocytosis, transfection, f-mating, electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile

30 bombardment; lipofection; and infection (where the vector is infectious, for instance,

a retroviral vector). The choice of introducing vectors or polynucleotides will often depend on features of the host cell.

Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell

5   genome. Amplified DNA can be isolated from the host cell by standard methods. See, e.g., Sambrook et al. (1989). RNA can also be obtained from transformed host cell, or it can be obtained directly from the DNA by using a DNA-dependent RNA polymerase.

Expression vectors generally are replicable polynucleotide constructs that

10   contain a polynucleotide encoding the polypeptide of interest. Herein, this means any of the MOR-1 splice variant polypeptides. For expression, one or more translational controlling elements are also usually required, such as ribosome binding sites, translation initiation sites and stop codons. These controlling elements (transcriptional and translational) can be derived from the MOR-1 gene, or

15   heterologous (i.e., derived from other genes or other organisms). A number of expression vectors suitable for expression in eukaryotic cells including yeast, avian, and mammalian cells are well known in the art. One example of an expression vector is pcDNA3 (Invitrogen, San Diego, CA), in which transcription is driven by the cytomegalovirus (CMV) early promoter/enhancer. This vector also contains

20   recognition sites for multiple restriction enzymes for insertion of an MOR-1 splice variant polypeptide of interest. Another example of an expression vector system is the baculovirus/insect system.

Cloning and expression vectors typically contain a selectable marker (for example, a gene encoding a protein necessary for the survival or growth of a host

25   cell transformed with the vector), although such a marker gene can be carried on another polynucleotide sequence co-introduced into the host cell. Only those host cells into which a selectable gene has been introduced will grow under selective conditions. Typical selection genes either: (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate; (b) complement auxotrophic

30   deficiencies; or (c) supply critical nutrients not available for complex media. The choice of the proper marker gene will depend on the host cell, and appropriate genes

for different hosts are known in the art. Vectors also typically contain a replication system recognized by the host.

Suitable cloning vectors can be constructed according to standard techniques, or selected from a large number of cloning vectors available in the art. While the
5   cloning vector selected may vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, may possess a single target for a particular restriction endonuclease, or may carry marker genes. Suitable examples include plasmids and bacterial viruses, e.g., pUC18, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3
10 and pAT28. These and other cloning vectors are available from commercial vendors such as BioRad, Stratagene, and Invitrogen.

According to an embodiment of the invention, the vectors, e.g., *in vivo* expression vectors, are viral vectors. Viral vectors, e.g., viral expression vectors are advantageously: poxviruses, e.g. vaccinia virus or an attenuated vaccinia virus.
15 When the expression vector is a vaccinia virus, insertion site or sites for the polynucleotide or polynucleotides to be expressed are advantageously at the thymidine kinase (TK) gene or insertion site, the hemagglutinin (HA) gene or insertion site, the region encoding the inclusion body of the A type (ATI); see also documents cited herein, especially those pertaining to vaccinia virus. The insertion
20 site or sites for MVA virus area advantageously as in various publications, including Carroll M. W. et al., Vaccine, 1997, 15 (4), 387-394; Stittelaar K. J. et al., J. Virol., 2000, 74 (9), 4236-4243; Sutter G. et al., 1994, Vaccine, 12 (11), 1032-1040; and, in this regard it is also noted that the complete MVA genome is described in Antoine G., Virology, 1998, 244, 365-396, which enables the skilled artisan to use other
25 insertion sites or other promoters. When the expression vector is a poxvirus, the polynucleotide to be expressed is inserted under the control of a specific poxvirus promoter, e.g., the vaccinia promoter 7.5 kDa (Cochran et al., J. Virology, 1985, 54, 30-35), the vaccinia promoter I3L (Riviere et al., J. Virology, 1992, 66, 3424-3434), the vaccinia promoter HA (Shida, Virology, 1986, 150, 451-457), the cowpox
30 promoter ATI (Funahashi et al., J. Gen. Virol., 1988, 69, 35-47), the vaccinia promoter H6 (Taylor J. et al., Vaccine, 1988, 6, 504-508; Guo P. et al. J. Virol.,

1989, 63, 4189-4198; Perkus M. et al., J. Virol., 1989, 63, 3829-3836), *inter alia*. When the expression vector is a herpes virus, the polynucleotide to be expressed is inserted under the control of a eukaryotic promoter, such as a strong eukaryote promoter, advantageously a CMV-IE (murine or human) promoter; that is, in

5   embodiments herein, the polynucleotide to be expressed is operably linked to a promoter, and in herpes virus embodiments, advantageously the polynucleotide to be expressed is operably linked to a strong eukatyotic promoter such as a mCMV-IE or hCMV-IE promoter.

The invention further encompasses an isolated polynucleotide, or a

10   complementary strand thereto that hybridizes under stringent conditions, comprising at least 15 consecutive nucleotides of the MOR-1 splice variant polynucleotides depicted in FIG. 2 where the polynucleotide contains promoter elements.

The MOR-1 splice variant promoter elements are contained in exons 1a, 1b, and 1c or in any combination thereof. Promoter elements can control the level,

15   tissue specificity, inducibility and, in gene clusters, the sequence of transcriptional activation and repression. Promoter elements include but are not limited to, enhancer sequences and repressor sequences.

The invention encompasses splice variant polypeptides. The exemplary MOR-1 splice variant polypeptides are composed of the amino acids indicated in

20   FIGS. 3 and 6. Polypeptide fragments comprising 5 amino acids, more preferably 7 amino acids, more preferably 15 amino acids, more preferably 25 amino acids, more preferably 50 amino acids and more preferably 75 amino acids, which are not the same as the known MOR-1 or MOR-1 variants are claimed herein and encompassed in the term "MOR-1 splice variant polypeptides".

25   The terms "protein", "peptide", "polypeptide" and "polypeptide fragment" are used interchangeably herein to refer to polymers of amino acid residues of any length. The polymer can be linear or branched, it may comprise modified amino acids or amino acid analogs, and it may be interrupted by chemical moieties other than amino acids. The terms also encompass an amino acid polymer that has been

30   modified naturally or by intervention; for example disulfide bond formation,

glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling or bioactive component.

The MOR-1 splice variant polypeptides retain MOR-1 activity. To "retain MOR-1 activity" is to have a similar level of functional activity as the MOR-1

5   polypeptide. This activity includes, but is not limited to, immunologic and pharmacologic activity.

The "immunologic activity" is binding to anti-opioid receptor antigen binding fragments. The antigen binding fragments can be any functional antibody, fragment or derivative thereof, including, but not limited to, whole native antibodies,

10   bispecific antibodies, chimeric antibodies, Fab, F(ab')2, single chain V region fragments (scFv), and fusion polypeptides comprising an antigen binding fragment fused to a chemically functional moiety.

The "pharmacologic activity" is activation or deactivation of the MOR-1 splice variant polypeptides upon binding of agonists or antagonists.

15   The invention further encompasses MOR-1 splice variant polypeptide homologs. A "homolog" is a polypeptide similar in amino acid sequence to other polypeptides among a single species or, a "homolog" in evolution is a polypeptide similar in amino acid sequence to other polypeptides in different species because they have been inherited from a common ancestor. Preferably, homologs of the

20   present invention are human homologs.

Isolation of MOR-1 splice variant human homolog cDNAs can be carried out by any method known in the art. For instance, methods analogous to the isolation of the human and rat MOR-1 splice variants described herein (see Examples 1 and 2). Using primers corresponding to the human MOR-1 gene and a Marathon-Ready

25   human cDNA Library to carry out reactions according to the Marathon cDNA Amplification Kit (Clontech), human MOR-1 splice variants can be obtained. Alternatively, screening of human cDNA libraries with probes corresponding to mouse MOR-1 splice variant sequences can be carried out at reduced stringency to identify human MOR-1 splice variant cDNAs.

30   The invention further encompasses the MOR-1 splice variant polypeptides in a heterodimeric or homodimeric form. A "heterodimer" is a protein made up of

more than one kind of polypeptide. A "homodimer" is a protein made up of more than one kind of polypeptide.

Pharmaceutical compositions and treatment modalities can be detected by the methods of this invention. The MOR-1 splice variant polypeptide fragments and

5   MOR-1 splice variant nucleic acid sequences can be used in screening for compositions that alter variant activity. Compositions that selectively regulate the MOR-1 splice variant polypeptide fragments or selectively modulate physiological processes can be identified.

The invention further encompasses methods of screening compositions for

10   opioid activity by obtaining a control cell that does not express a recombinant opioid receptor and obtaining a test cell that is the same as the control cell except that it expresses a recombinant MOR-1 splice variant polypeptide, contacting the control cell and test cell with an amount of an opioid sufficient to exert a physiologic effect, separately measuring the physiologic effect of the composition on the control cell

15   and test cell and comparing the physiologic effect of the composition to the physiologic effect of the opioid, where determination of a physiologic effect of the composition is expressed relative to that of the opioid.

The invention further comprises a method of screening compositions for opioid activity by obtaining a control polypeptide that is not a recombinant opioid

20   receptor and obtaining a test polypeptide that is a recombinant MOR-1 splice variant polypeptide, contacting a composition with the control polypeptide and the test polypeptide, contacting the test polypeptide with an amount of an opioid sufficient to measurably bind the test polypeptide, measuring the binding of the composition and the opioid and comparing the test polypeptide binding of the composition to that

25   of the opioid, where determination of binding of the composition is expressed relative to that of the opioid.

The invention further encompasses a method of screening compositions for differential opioid activity by obtaining a first test polypeptide that is an MOR-1 splice variant polypeptide and contacting it with a composition and obtaining a

30   second test polypeptide that is an MOR-1 splice variant polypeptide, measuring the binding of the composition to the first and second test polypeptides, and comparing

the binding of the composition and the first test polypeptide to that of the second test polypeptide where differential activity is expressed as a ratio of the two binding affinities.

The invention further encompasses methods of screening compositions for a
5   MOR-1 binding protein by obtaining a control cell that does not express a recombinant or endogenous opioid receptor, obtaining a test cell that expresses a recombinant MOR-1 splice variant polypeptide, contacting the control cell extract and test cell extract with the MOR-1 splice variant polypeptide and comparing the test cell extract binding to that of the control cell extract binding with the MOR-1
10   splice variant polypeptide of interest to identify a MOR-1 binding protein. For example, but not by limitation, the MOR-1 binding protein can be identified by two hybrid screen, which is known to one of skill in the art (see, e.g., U.S. Patent Nos. 5,525,490; 5,948,620; 5,955,280; 5,965,368; 6,051,381; 6,251,676; 6,479,289 and 6,562,576).

15   The compositions screened include, but are not limited to, chemical, synthetic combinatorial libraries of small molecule ligands, eukaryotic whole cell lysates or extracts, media conditioned by cultured eukaryotic cells, natural products and extracts thereof.

The opioid can be, but is not limited to, morphine, methadone, etorphine,
20   levorphanol, fentanyl, sufentanil, [D-Ala^2, MePhe^4, Gly(ol)^5]enkephalin (DAMGO), pentazocine, ethylketocyclazocine, bremazocine, spiradoline, [D-Ser^2, Leu^5]enkephalin-Thr^6 (DSLET), Met-enkephalin, Leu-enkephalin, β-endorphin, dynorphin A, dynorphin B, α-neoendorphin analogs and combinatorial chemistry products thereof.

25   The physiological effect can be measured by any method known in the art such as changes in the levels of neuroendocrine hormones, including, but not limited to prolactin, growth hormone, gonadotropin-releasing hormone, adrenocorticotropin, corticotropin-releasing factor, luteinizing hormone, follicle stimulating hormone, testosterone or cortisol. The physiological effect can also be measured by changes
30   in the levels of neurotransmitters, including but not limited to, acetylcholine or dopamine.

Activation of an MOR-1 receptor, and likely, the MOR-1 splice variant polypeptides, stimulates a variety of physiological responses, including analgesia, depression of gastrointestinal motility and respiration, and alterations of the immune, endocrine and autonomic nervous system. Compositions that regulate the activity of the MOR-1 receptor and/or the MOR-1 splice variant polypeptides can elicit responses that have therapeutic effects. The invention is useful in diagnosis, treatment, design and screening of novel reagents. Screening of compounds can result in obtaining those with differential or selective activity. That is, for instance, certain compositions can retain analgesic effects but do not affect peristaltic activity and thus do not cause constipation. Conversely, compositions that lack analgesic effects but affect peristaltic activity would be useful in treating chemotherapy and HIV patients. Other applications relating to the side effects of opiates can be readily envisaged by one of skill in the art.

The invention further encompasses a method for regulating morphine analgesia in a subject by altering the amount of MOR-1 splice variant polypeptide activity in the subject. Activity can be regulated by administering antigen binding fragments, agonists, antagonists or small molecule ligands to a subject in an amount and duration sufficient to regulate morphine analgesia. The antigen binding fragment, agonist, antagonist or small molecule ligand is directed to an MOR-1 splice variant. Activity can also be regulated by homodimerization or homooligomerization among the MOR-1 splice variants and heterodimerization or heterooligomerization between the variants and other opioid or non-opioid receptors.

Activity can also be regulated by administering a DNA plasmid vector containing an MOR-1 splice variant polynucleotide. The DNA plasmid vector thereby expresses an MOR-1 splice variant polynucleotide in a subject in an amount and a duration sufficient to regulate morphine analgesia. Activity can also be regulated by administering an antisense nucleic acid or siRNA complementary to an MOR-1 splice variant polynucleotide, thereby blocking gene expression in a subject in an amount and a duration sufficient to regulate morphine analgesia. Methods for administering antisense and siRNA are known to one of skill in the art (reviewed in

Brantl, Biochim Biophys Acta. 2002 May 3;1575(1-3):15-25 and Lavery & King, Curr Opin Drug Discov Devel. 2003 Jul;6(4):561-9).

Agonists and antagonists of MOR-1 splice variant polypeptide activity can include but are not limited to, morphine, methadone, etorphine, levorphanol,

5   fentanyl, sufentanil, [D-Ala^2, MePhe^4, Gly(ol)5]enkephalin (DAMGO), butorphanol, naloxone, naltrexone, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$ (CTOP), diprenorphine, β-funaltrexamine, naloxonazine, nalorphine, pentazocine, nalbuphine, benzoylhydrazone, bremazocine, ethylketocyclazocine, trans-(-)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (also known

10   as U50488), (5-alpha,7-alpha,8-beta)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzeneacetamide (also known as U69593), spiradoline, naltrindole, [D-Pen^2, D-Pen-^{5}]enkephalin (DPDPE), [D-Ala^2,Glu^4]deltorphin, [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET), Met-enkephalin, Leu-enkephalin, β-endorphin, dynorphin A, dynorphin B, α-neoendorphin and derivatives such as those produced

15   by combinatorial chemistry and their mixtures and physiologically acceptable salts thereof.

A "subject" is a vertebrate, preferably a mammal, and more preferably a human. Mammals include but are not limited to humans, farm animals, sport animals, and pets.

20   The invention further encompasses antigen binding fragments specific for an MOR-1 splice variant polypeptide. According to the invention, an MOR-1 splice variant polypeptide can be used as an immunogen to generate antigen-binding fragments which immunospecifically bind the immunogen.

Production of antigen binding fragments such as polyclonal antibodies can

25   be carried out by any method known in the art. Various host animals can be immunized by injection with the immunogen, including but not limited to rabbits, mice and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete or incomplete) adjuvant, mineral gels such as aluminum hydroxide,

30   surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially

useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

For preparation of antigen binding fragments such as monoclonal antibodies, any technique that provides for the production of antibody molecules by continuous

5   cell lines in culture can be used. Examples of such techniques include the original hybridoma technique (Kohler and Milstein (1975) Nature 256:495) as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al. (1983) Immunol. Today 4:72), and the EBV hybridoma technique to produce human monoclonal antibodies. Cole et al. (1985) in Monoclonal Antibodies and Cancer

10   Therapy, Alan R. Liss, Inc., pp. 77-96. Monoclonal antibodies can also be produced in germ-free animals utilizing known technology. PCT/US90/02545. Human antibodies can be obtained using human hybridomas (Cote et al. (1983) Proc. Natl. Acad. Sci. U.S.A. 80:2026), or by transforming human B cells with EBV virus in vitro. Cole et al. (1985). Techniques developed for the production of "chimeric

15   antibodies" by splicing the genes from a mouse antibody molecule specific for MOR-1 splice variants together with genes from a human antibody of appropriate biological activity can be used. Morrison et al. (1984) Proc. Natl. Acad. Sci. U.S.A. 81:6851; Neuberger et al. (1984) Nature 312:604; and Takeda et al. (1985) Nature 314:452.

20   Techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce MOR-1 splice variant polypeptide-specific single chain antibodies. Techniques described for the production of Fab expression libraries (Huse et al. (1989) Science 246:1275) can be utilized, allowing rapid and easy identification of monoclonal Fab fragments specific for an MOR-1

25   splice variant polypeptide.

Antibody fragments that contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(abl), fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the

30   disulfide bridges of the F(abl) fragment, the Fab fragments which can be generated

by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

Single chain V region fragments ("scFv") can also be produced. Single chain V region fragments are made by linking L (light) and/or H (heavy) chain V

5 (variable) regions by using a short linking peptide. Bird et al. (1988) Science 242:423. Any peptide having sufficient flexibility and length can be used as a linker in a scFv. Usually the linker is selected to have little to no immunogenicity. An example of a linking peptide is (GGGGS)$_3$, (SEQ ID NO: 89) which bridges approximately 3.5 nm between the carboxy terminus of one V region and the amino

10 terminus of another V region. Other linker sequences can also be used, and can provide additional functions, such as for attaching a drug or a solid support.

All or any portion of the H or L chain can be used in any combination. Typically, the entire V regions are included in the scFv. For instance, the L chain V region can be linked to the H chain V region. Alternatively, a portion of the L chain

15 V region can be linked to the H chain V region or a portion thereof. Also contemplated are scFvs in which the H chain V region is from H11, and the L chain V region is from another immunoglobulin. It is also possible to construct a biphasic, scFv in which one component is an MOR-1 splice variant polypeptide and another component is a different polypeptide, such as a T cell epitope.

20 The scFvs can be assembled in any order, for example, V_H—(linker)—V_L or V_L—(linker)—V_H. There may be a difference in the level of expression of these two configurations in particular expression systems, in which case one of these forms may be preferred. Tandem scFvs can also be made, such as (X)—(linker)—(X)—(linker)—(X), in which X are MOR-1 splice variant

25 polypeptides, or combinations of MOR-1 splice variant polypeptides with other polypeptides. In another embodiment, single chain antibody polypeptides have no linker polypeptide, or just a short, inflexible linker. Exemplary configurations include V_L—V_H and V_H—V_L. The linkage is too short to permit interaction between V_L and V_H within the chain, and the chains form homodimers with a V_L/V_H antigen-

30 binding site at each end. Such molecules are referred to in the art as "diabodies".

ScFvs can be produced either recombinantly or synthetically. For synthetic production of scFv, an automated synthesizer can be used. For recombinant production of scFv, a suitable plasmid containing a polynucleotide that encodes the scFv can be introduced into a suitable host cell, either eukaryotic, such as yeast,

5 plant, insect or mammalian cells, or prokaryotic, such as *Escherichia coli*, and the protein expressed by the polynucleotide can be isolated using standard protein purification techniques.

A particularly useful system for the production of scFvs is plasmid pET-22b(+) (Novagen, Madison, WI) in *E. coli*. pET-22b(+) contains a nickel ion

10 binding domain consisting of 6 sequential histidine residues, which allows the expressed protein to be purified on a suitable affinity resin. Another example of a suitable vector is pcDNA3 (Invitrogen, San Diego, CA), described above.

The following examples are provided to illustrate but not limit the claimed invention. The examples demonstrate isolation and characterization of MOR-1

15 splice variants, and are representative of the methods employed for all claimed MOR-1 splice variants.

EXAMPLE 1: Identification and characterization of six new alternatively spliced variants from the human mu opioid receptor gene, *Oprm*

The mu opioid receptor plays an important role in mediating the actions of morphine and morphine-like drugs. Binding and pharmacological studies have

5   proposed several mu receptor subtypes, but only one mu opioid receptor (*Oprm*) gene has been isolated. The Examples herein describe the identification and characterizion of six new splice variants from the human *Oprm* gene using an RT-PCR strategy. The variants display differences on their carboxyl termini resulting from alternative splicing of the fourth exon. Northern blot analysis demonstrated

10   expression of the variant mRNAs. Receptor binding assays established that these variants belonged to the mu opioid receptor family with limited differences in opioid ligand affinities. However, adenylyl cyclase assays revealed significant differences in both opiate potency and efficacy among these variants. Dissociation between binding affinity and efficacy/potency among these variants might provide important

15   insights to understanding the varied opioid responses observed clinically, and into designing new selective drugs based upon these differences in efficacy/potency.

Primer design. In order to identify potential mouse homologs containing exons 5 in the human *Oprm* gene, a sequence alignment was performed among a ~ 7.7 kb mouse genomic sequence containing exons 5a, 5b, 5c, 5d and 5e, a ~ 7.3 kb

20   rat and a ~ 8 kb human corresponding genomic sequences obtained from Public human genome databases (NBCI and Ensembl), with Vector NTI software (Informax). Three sets of antisense primers were designed. One of the primers (primer A) was derived from the human exon 5a that was highly homologous to the mouse or rat exon 5a, while the other two sets of primers (primers B and C) were

25   from the regions homologous to the mouse exons 5c and 5d, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR) cloning. Total RNA was extracted from Be(2)C, a human neuroblastoma cell line, with the guanidinium thiocyanate phenol-chloroform extraction method (see, e.g., Pan et al. (1995) Molecular Pharmacology 47, 1180-1188), and reverse transcribed with

30   primer A (5'-GCT TCC AAT CTT ATA TTC TTT CAC GG-3', SEQ ID NO:1) and Superscript II reverse transcriptase (Invitrogen) as previously described. The first-strand cDNA was then used as a template in PCRs with a sense primer A1 from the

5' untranslated region of exon 1 (5'-GAA AGG AAG CGG CTG AGG CGC T-3', SEQ ID NO:2) and the antisense primer A from exon 5a, or the antisense primer B1 from exon 5c (5'-GTG TAT TGT CTA TTA GAG TGA GGC TAA CAT TTC TTT GG-3', SEQ ID NO:3), or the antisense primer C1 from exon 5d (5'-CCA CAC

5   GGC AGT ACC TTC TCT TGG TCT CC-3', SEQ ID NO:4). Having no visible bands by the first-round PCR, nested PCRs were followed by using the first-round PCR products as templates and nested primers including a sense primer A2 (5'-CGG TGC TCC TGG CTA CCT CGC A-3', SEQ ID NO:5) and the antisense primer A, or the antisense primer B2 (5'-GGT TAG ATG GCT TTT ATC ATC ATA TTG

10   CTG G-3', SEQ ID NO:6), or the antisense primer C2 (5'-GGG AAC AGG AAT TTT AGG GTT CAT GTCATA G-3', SEQ ID NO:7). Six cDNA fragments arranging from ~1.2 to 2.6 kb in size were obtained, subcloned into pCRII-TOPO vector (Invitrogen), and sequenced in both strands. Sequence analysis of the cDNAs showed that all the clones had the same exons 1, 2 and 3 as the original hMOR-1,

15   but contained an alternative fourth exon that was resulted from combination of six different exons (exons 5a, 5b, 5c, 5d, 5e and Y generated by using different splice site within exon 5 and a novel exon, exon Y). The corresponding variants were named as hMOR-1B1, hMOR-B2, hMOR-1B3, hMOR-1B4, hMOR-1B5 and hMOR-1Y, respectively. hMOR-1A was amplified from the first-strand cDNA

20   reverse-transcribed from Be(2)C RNA with random primers in a nested PCR using two sense primers (A1 & A2) from exon1 and two antisense primers (D1: 5'-GCT TCC CCT CTT CCC TCC ATT CTC-3', SEQ ID NO:8; D2: 5'-GGA TTA AAC TCC TAG TTT AGC ACA AAG CC-3', SEQ ID NO:9) from exon 3b sequence obtained from Public genome databases.

25   Northern blot analysis. Northern blot analysis was performed as described previously (see, e.g., Pan et al. (2001) Proc.Natl.Acad.Sci.U.S.A 98, 14084-14089). In brief, total RNA was isolated from Be(2)C cells (see above). 20 μg of total RNA per lane was loaded, separated on a 0.8% formaldehyde agarose gel, and transferred to a GenePlus membrane. The membrane was then hybridized with ^{32}P-labeled

30   cDNA probes generated by PCR with appropriate primers. The primers used were a sense primer (E: 5'-GCC ACC AGT ACC CTG CCC TTC C-3', SEQ ID NO:10)

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from exon 2 and an antisense primer (F: 5'-CTC AAT GTT GGA AGA GGT TGG GAT AC-3', SEQ ID NO:11) from exon 3 for a exons 2&3 probe, a sense primer (G: 5'-GTA CGC AGT CTC TAG AAT TAG GTA TAT CTA CTG-3', SEQ ID NO: 12) and an antisense primer (H: 5'-GGA TTC TAG ATC AGA ATT ATT TCT

5 ATA ATG TGC-3', SEQ ID NO:13) from exon 3b for an exon 3b probe, a sense primer (I: 5'-GAG ACC ACC CCT CCA CGG C-3', SEQ ID NO: 14) from exon 3a and an antisense (J: 5'-GGT CTC CAT TAG GGC TAG CAG CAG-3', SEQ ID NO:15) from exon 5a for na exon 5a probe, a sense primer (K: 5'-CAG AGA GAA AGA AGA CAG AAA TCT GAC TGG TAA G-3', SEQ ID NO:16) and an

10 antisense primer (L: 5'-GAG AGC ACG TGT TGA AAC TGC AAG TCA GAG-3', SEQ ID NO:17) from exon 5b for an exon 5b probe, a sense primer (M: 5'-GGA CCT CCA GCC AAG TTT GTT GCT GAC-3', SEQ ID NO:18) and an antisense primer (N: 5'-CTC TCT GTG CAA ACG GTT GAA TGA ATG G-3', SEQ ID NO:19) from exon 5c for an exon 5c probe, a sense primer (O: 5'-CAG AGC TGA

15 CTA TGA CAT GAA CCC TAA AAT TCC TG-3', SEQ ID NO:20) and an antisense primer (P: 5'-GGT CCC TGA AAC CAA CAA AAA AAC TGG ATG-3', SEQ ID NO: 21) from exon 5d for an exon 5d probe, a sense primer (Q: 5'-CAG GTG GAA TTG AAC CTG GAC TGT CAC TGT G-3', SEQ ID NO:22) and an antisense primer (S: 5'-GCT CTA AAA ATC ATA TGA AAT AGT TAC AAG

20 CCT TTG-3', SEQ ID NO:23) from exon 5e for an exon 5e probe, and a sense primer (T: 5'-CAT CAG ATC AGA GAT CCA ATA TCA AAC CTT CCC-3', SEQ ID NO:24) and an antisense primer (U: 5'-GGA GGT CCC TTG ATA ACT GCC AAA TCG C-3', SEQ ID NO:25) from exon Y for an exon Y probe. The sizes of the probes were 728 bp for exons 2&3 probe, 319 bp for exon 3b probe, 184 bp

25 for exon 5a probe in which a 47 bp sequence was from exon 3a, 309 bp for exon 5b probe, 272 bp for exon 5c probe, 145 bp for exon 5d probe, 156 bp for exon 5e probe, and 122 bp for exon Y probe, respectively. After washing, the membranes were exposed to Kodak BioMax MS film.

Expression of the variants in Chinese Hamster Ovary (CHO) cells. The

30 cDNA fragments containing full-length variants including the original hMOR-1 in pCRII-TOPO were subcloned into pcDNA5FRT (Invitrogen) or pcDNA3 vector. A

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cDNA fragment containing only exons 1, 2 and 3 was also generated by PCR with the sense primer A2 from exon 1 (see above) and an antisense primer (V: 5'-GAT CTC GAG TCA TTA CTG ATG ATT AGT TCT ATC CAC TGT ATT GGC-3, SEQ ID NO:26) and subcloned into pcDNA5FRT. The resulting plasmids, hMOR-

5 1/pcDNA5FRT, hMOR-1A/pcDNA5FRT, hMOR-1B1/pcDNA3, hMOR-1B2/pcDNA5FRT, hMOR-1B3/pcDNA5FRT, hMOR-1B4/pcDNA5FRT, hMOR-1B5/pcDNA5FRT, hMOR-1Y/pcDNA5FRT and hMOR-1(exons1-3)/pcDNA5FRT were transfected into CHO cells with or without pOG44 construct (Flip-In system, Invitrogen) by LipofectAMINE reagent (Invitrogen). Stable transformants were

10 obtained 10 - 14 days after selection with hygromycin or G418 and screened with [^{3}H]DAMGO binding assay.

Receptor Binding Assays. Membranes were prepared from stable transfectants as described previously (see, e.g., Pan et al. (1999) Molecular Pharmacology 56, 396-403). [^{3}H]DAMGO saturation and competition binding

15 assays were performed at 25°C for 60 minutes in 50mM potassium phosphate buffer, pH 7.4, containing 5mM magnesium sulfate. Specific binding was defined as the difference between total binding and nonspecific binding, determined in the presence of levallorphan (10 μM). K_D, Bmax and K_i values were calculated by nonlinear regression analysis (GraphPad Prism, Carslbad, CA). Protein

20 concentrations were determined as previously described using BSA as the standard (see, e.g., Lowry et al. (1951) Journal of Biological Chemistry 193, 265-275).

Adenylyl cyclase assay. Adenylyl cyclase activity in intact cells was determined as previously reported (Thakker et al. (2003) Methods Mol Med. 84, 29-37). Briefly, intact stable transfectant cells (0.07-0.18 mg of protein) were incubated

25 in Hanks' balanced salt solution (137 mM NaCl, 5 mM KCl, 0.6 mM Na$_2$HPO$_4$, 0.4 mM KH$_2$PO$_4$, 4 mM NaHCO$_3$, 6 mM D-glucose, 0.5 mM MgCl$_2$, 0.4 mM MgSO$_4$, and 1 mM CaCl$_2$) containing 0.5 mM 3-isobutyl-1-methylxanthine for 5 min at 37°C. after adding forskolin (10 μM) and/or agonists (0.01nM - 10μM) on ice, cells were then incubated for 10 min at 37°C. The reaction was stopped by incubating

30 the tubes in a boiling water bath for 6 min. The tubes were centrifuged for 5 min at 11,000g, and cAMP levels from the supernatant were then measured in a

competition assay for [^{3}H]cAMP binding. The supernatants were incubated with 0.8

pM [^{3}H]cAMP in binding buffer containing 25 mM Tris-HCl, pH 7.0, 10 mM

theophylline, 0.1% BSA, , and 0.25 mg/ml adrenal cortex extract for 1 h at 4°C.

After adding 75 μl of hydroxyapatite [50% (w/v)] and incubating for 6 min at 4°C to

5   terminate the reaction, the reaction was then filtered through No. 34 glass-fiber

filters (Schleicher & Schuell) and washed three times with 3 ml of ice-cold 10 mM

Tris-HCl, pH 7.0 on a semiautomatic cell harvester. Filters were transferred into

vials with 5 ml of Liquiscent (National Diagnostics, Atlanta, GA), and the

radioactivity in vials were determined by scintillation spectroscopy in a Packard

10   TRI-CAEB 2900TR counter. A parallel standard curve setting using unlabeled

cAMP (0.4 – 125 pmol) was performed to calculate the cAMP level in the

supernatants.

Cloning new splice variants from the human Oprm Gene. To isolate

potential human MOR-1 variants homologous to those identified in mice, three sets

15   of human antisense primers were designed based upon the genomic sequence

alignment among mouse, rat and human, and then used these antisense primers

together with two sense primers from 5'UTR of exon 1 in an RT-PCR strategy. Six

new variants were identified, hMOR-1B1, hMOR-1B2, hMOR-1B3, hMOR-1B4,

hMOR-1B5 and hMOR-1Y, from Be(2)C, a human neuroblastoma cell line that

20   expresses high level of mu opioid receptors (FIG. 1A). Sequence analysis revealed

that all the variants contained the same exons 1, 2 and 3 as the original MOR-1, but

had a different fourth exon, which resembled splicing patterns similar to two earlier

human (hMOR-1O and hMOR-1X) as well as eleven mouse (mMOR-1B1, mMOR-

1B2, mMOR-1B3, mMOR-1B4, mMOR-1B5, mMOR-1C, mMOR-1D, mMOR-1E,

25   mMOR-1F, mMOR-1O and mMOR-1P) variants (see, e.g., Pan et al. (2000) FEBS

Letters 466, 337-340 and Suzuki et al. (1998) Life Sciences 64, PL1-PL7).

Therefore, all these variants contained the same protein structure on cell membrane,

except with a different intracellular carboxyl terminus (FIG. 2). The common

protein sequence includes all seven transmembrane domains and the binding pocket.

30   Similar to the mouse variants associated with exons 5 (mMOR-1B1, mMOR-1B2,

mMOR-1B3, mMOR-1B4 and mMOR-1B5), the human exon 5 variants were

produced from alternative splicing within exon 5 and all the splice junctions contained consensus splicing sequences. However, the predicted amino acid sequences from the human variants differed from those from the mouse variants, and from each other due to sequence divergence (Figs. 1B and 2).

5   In hMOR-1B1, the first five of the total eighteen amino acids deduced from exon 5a were identical to those in mMOR-1B1 and rat MOR-1B. However, there was no termination codon after the fifth amino acid as in mMOR-1B1 and rMOR-1B, and translation stopped after the eighteenth amino acid. Therefore, hMOR-1B1 contained an additional thirteen amino acids on its carboxyl terminal. Exons 5b, 5c,

10   5e and Y predicted 9, 15, 22 and 14 amino acids in hMOR-1B2, hMOR-1B3, hMOR-1B5 and hMOR-1Y, respectively, whereas exon 5d in hMOR-1B4 deduced only one amino acid, a serine residue, the shortest version of all carboxyl terminal variants cloned so far. Several potential protein kinase phosphorylation sites for cAMP- and cGMP-dependent protein kinase and tyrosin kinase were present in

15   hMOR-1B1, hMOR-1B2 and hMOR-1Y, respectively (FIGS. 1B and 2).

          Northern blot analysis of human MOR-1 variant mRNA. Northern blot analysis was performed to estimate the relative size and abundance of the variant mRNAs. Since all the variants contained exons 1, 2 and 3, the exons 2/3a probe was designed as a control to define all the variants. It detected several diffuse and heavy

20   bands ranging from 2 to 15 kb. A similar band pattern was also seen in the mouse and rat Northern blots with their exons 2/3a probes. The exon 5a probe hybridized a strong band around 12 kb, which had a similar size and an intensity when compared to the higher band detected by the exon 4 probe that defines the original human MOR-1. This similarity implied that there was a relatively high abundance for the

25   variants associated with exon 5a. The exon 5a probe also hybridized several light diffuse bands at approximately 15, 3.5 to 5 and 2 to 3 kb, respectively, suggesting that exon 5a may be associated with multiple variants. However, the exons 5b, 5c, 5d, 5e and Y probes as well as exon 3b probe all detected a well-defined band at about 15 kb, suggesting that they may share a similar RNA structure, particularly in

30   the 3'UTR, even if the protein sequences are different. The exon Y probe also hybridized two distinct bands at approximately 12 and 3.5 kb. Although the smaller

one may be a degradation product, it also is possible that exon Y may be associated with more than one variant.

Table 1 depicts the identity and homology among the human mu opioid receptor variants.

5   Table 2 presents the saturation studies with [^{3}H]DAMGO. [^{3}H]DAMGO binding was performed in membranes isolated from stable transfectants containing the indicated cDNA clones. The binding parameters were established by nonlinear regression analysis. Results are the mean ± S.E.M of at least three independent determinations.

10   Table 3 presents the competition of [^{3}H]DAMGO binding among the human MOR-1 variants. Competition studies against [^{3}H]DAMGO (~1nM) were performed with indicated ligands using at least three concentrations of drugs and the Ki value calculated as previously described. Results are the means ± S.E.M. of three independent determinations. The ligands that showed significant differences among

15   the variants using ANOVA were DAMGO ($p < 0.0262$), Morphine ($p < 0.0359$), M6G ($p < 0.0003$), DSLET ($p < 0.0027$), endomorphin 1 ($p < 0.0068$), endomorphin 2 ($p < 0.0084$), β-endorphin ($p < 0.0005$). For DAMGO, Tukey determined differences between hMOR-1(exons 1-3) and hMOR-1B2, hMOR-1B1 and hMOR-1B2, hMOR-1B3 and hMOR-1B2 ($p < 0.05$). For morphine, Tukey determined differences

20   between hMOR-1(exons 1-3) and hMOR-1B2, hMOR-1B1 and hMOR-1B2, hMOR-1B2 and hMOR-1B3 ($p < 0.05$). For M6G, Tukey determined differences between hMOR-1 and hMOR-1B2, hMOR-1(exons 1-3) and hMOR-1B2, hMOR-1A and hMOR-1B2, hMOR-1B1 and hMOR-1B2, hMOR-1B2 and hMOR-1B3($p < 0.01$). For DSLET, Tukey determined differences between hMOR-1 and

25   hMOR-1B2($p < 0.05$), hMOR-1(exons 1-3) and hMOR-1B2 ($p < 0.01$), hMOR-1A and hMOR-1B2 ($p < 0.05$), hMOR-1B1 and hMOR-1B2, hMOR-1B2 and hMOR-1B3, hMOR-1B2 and hMOR-1B5, hMOR-1B2 and hMOR-1Y ($p < 0.01$). For endomorphin 1, Tukey determined differences between hMOR-1 and hMOR-1B2, hMOR-1(exons 1-3) and hMOR-1B2, hMOR-1B1 and hMOR-1B2 ($p < 0.05$). For

30   endomorphin 2, Tukey determined differences between hMOR-1 and hMOR-1(exons 1-3), hMOR-1 and hMOR-1A, hMOR-1 and hMOR-1B1, hMOR-1 and

hMOR-1B3 (p<0.05). For β-endorphin, Tukey determined differences between hMOR-1 and hMOR-1B2, hMOR-1(exons 1-3) and hMOR-1B2, hMOR-1A and hMOR-1B2, hMOR-1B1 and hMOR-1B2, hMOR-1B2 and hMOR-1B3 (p<0.01).

Table 4 depicts saturation studies with [^{3}H]DAMGO. [^{3}H]DAMGO binding
5   was performed in membranes isolated from stable transfectants containing the indicated rat cDNA clones. The binding parameters were established by nonlinear regression analysis. Results are the mean ± S.E. of at least three independent determinations.

Table 5 present the relative efficacy of opioid ligands for hMOR-1 and
10   hMOR-1 variants in adenylyl cyclase assay. The highest level of inhibition for any of the drugs (Table 4) for a specific variant was arbitrarily defined as 100%. Efficacy for all the other compounds for the indicated variant was defined as the percentage of the maximal inhibition for that variant.

Table 6 depicts the inhibition of forslolin-stimulated cAMP accumulation by
15   opioids in hMOR-1 variants. The IC$_{50}$ and maximal inhibition were calculated by nonlinear regression analysis (Prism 3.0). Results are the means ± S.E.M. of at least three independent determinations. Significant differences of maximal inhibition analyzed by ANOVA were DAMGO (p<0.0001), and morphine (p<0.0001). For maximal inhibition by DAMGO, post hoc analysis using Tukey revealed significant
20   differences between hMOR-1 and hMOR-1B1 (p,0.05), hMOR-1A and hMOR-1B1 (p<0.01), hMOR-1B1 and hMOR-1B2 (p<0.001), hMOR-1B1 and hMOR-1B5 (p<0.01), hMOR-1B1 and hMOR-1B6 (p<0.001), hMOR-1B2 and hMOR-1B4 (p<0.01), hMOR-1B3 and hMOR-1B6 (p<0.05), hMOR-1B4 and hMOR-1B6 (p<0.01). For maximal inhibition by morphine, Tukey determined significant
25   differences between hMOR-1 and hMOR-1B3, hMOR-1(exons 1-3) and hMOR-1B3, hMOR-1A and hMOR-1B3 (p<0.001), hMOR-1B1 and hMOR-1B6 (p<0.01), hMOR-1B2 and hMOR-1B4 (P<0.05), hMOR-1B3 and hMOR-1B4, hMOR-1B4 and B6 (p<0.01), hMOR-1B3 and hMOR-1B5, hMOR-1B3 and hMOR-1B6 (p<0.001). Significant differences of IC$_{50}$ by ANOVA were DAMGO (p<0.0008),
30   morphine (p<0.0002), β-endorphin (p<0.0001). For IC$_{50}$ by DAMGO, Tukey determined significant differences between hMOR-1 and hMOR-1B1, hMOR-1B1

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and hMOR-1(exons 1-3) ($p<0.01$), hMOR-1 and hMOR-1B3, hMOR-1(exons 1-3) and hMOR-1B3, hMOR-1A and hMOR-1B1, hMOR-1B1 and hMOR-1B6 ($p<0.05$). For IC_{50} by morphine, Tukey determined significant differences between hMOR-1 and hMOR-1B2, hMOR-1(exons 1-3) and hMOR-1B2 ($p<0.001$), hMOR-1A and

5   hMOR-1B2, hMOR-1B2 and hMOR-1B3, hMOR-1B2 and hMOR-1B4, hMOR-1B2 and hMOR-1B5, hMOR-1B2 and hMOR-1B6 ($p<0.01$). For IC_{50} by β-endorphin, Tukey determined significant differences between hMOR-1 and hMOR-1B5, hMOR-1A and hMOR-1B5, hMOR-1B1 and hMOR-1B5, hMOR-1B5 and hMOR-1B6 ($p<0.001$), hMOR-1B3 and hMOR-1B5, hMOR-1B4 and hMOR-1B5

10   ($p<0.01$)

Table 1

Identity (Homology)	hMOR-1 (400)	hMOR-1A (392)	hMOR-1B1 (406)	hMOR-1B2 (397)	hMOR-1B3 (403)	hMOR-1B4 (389)	hMOR-1B5 (410)	hMOR-1O (418)	hMOR-1X (446)	hMOR-1Y (402)
hMOR-1 (400)										
hMOR-1A (392)	97.3 (99.3)									
hMOR-1B1 (406)	96.1 (97.5)	95.8 (99.3)								
hMOR-1B2 (397)	97.3(98.0)	97.7 (99.0)	96.1 (98.3)							
hMOR-1B3 (403)	96.5 (97.3)	97.7 (99.0)	95.8 (96.6)	96.5 (98.0)						
hMOR-1B4 (389)	96.8 (99.5)	98.7 (99.5)	95.3 (99.5)	97.5 (99.5)	96.0 (99.5)					
hMOR-1B5 (410)	95.6 (98.0)	94.5 (99.3)	94.4 (97.3)	95.1 (98.3)	94.9 (96.6)	94.4 (99.5)				
hMOR-1O (418)	92.8 (97.1)	92.8 (99.0)	92.8 (95.7)	92.8 (97.8)	93.5 (97.1)	92.6 (99.5)	93.3 (95.2)			
hMOR-1X (446)	87.0 (97.3)	87.0 (99.1)	87.7 (96.6)	87.4 (98.4)	87.2 (96.9)	86.8 (99.6)	87.7 (95.7)	87.4 (93.7)		
hMOR-1Y (402)	96.5 (97.0)	96.8 (99.3)	95.8 (96.8)	96.5 (97.8)	96.3 (96.5)	96.3 (99.5)	94.6 (96.6)	93.1 (96.9)	87.2 (97.1)	

Table 2

Clone	K_D (nM)	B_{max} (pmol/mg protein)
hMOR-1	1.6 ± 0.6	1.01 ± 0.20
hMOR-1(exons 1-3)	1.2 ± 0.3	0.66 ± 0.09
hMOR-1A	1.7 ± 0.6	1.04 ± 0.13
hMOR-1B1	3.1 ± 0.8	0.49 ± 0.08
hMOR-1B2	4.4 ± 0.4	0.37 ± 0.04
hMOR-1B3	1.6 ± 0.5	0.37 ± 0.02
hMOR-1B4	2.6 ± 0.2	0.56 ± 0.10
hMOR-1B5	1.4 ± 0.1	0.44 ± 0.08
hMOR-1Y	2.3 ± 0.6	0.69 ± 0.15

Table 3

Ligand	K_i value (nM)								
	hMOR-1wt	hMOR-1 w/o exon 4	hMOR-1A	hMOR-1B1	hMOR-1B2	hMOR-1B3	hMOR-1B4	hMOR-1B5	hMOR-1B6
DAMGO	2.0 ± 0.8	1.5 ± 0.4	2.4 ± 0.6	1.2 ± 0.7	5.8 ± 2.4	1.8 ± 0.9	2.3 ± 1.1	2.1 ± 0.7	2.5 ± 1.4
Morphine	4.2 ± 1.4	2.9 ± 0.9	4.4 ± 1.7	2.4 ± 2	11.2 ± 6.1	3.2 ± 1.1	5.5 ± 3	3.9 ± 1.6	4.3 ± 3
M6G	13.9 ± 3.2	11 ± 4.6	13.2 ± 6.6	5 ± 0.4	42.2 ± 13.7	15.7 ± 2.1	22.8 ± 12.9	12.2 ± 4.5	8.3 ± 3.9
DADLE	3.9 ± 1.3	4.5 ± 1.5	4.9 ± 1.1	3.6 ± 2.6	10 ± 5.9	4.6 ± 0.4	5.1 ± 2.1	6.8 ± 2.8	6.5 ± 3
DSLET	19.1 ± 5.8	14 ± 2	19.7 ± 7.7	13.9 ± 4.9	34.4 ± 6.4	16.4 ± 2.8	22.3 ± 2	16 ± 6.6	15.4 ± 3.5
Naloxone	2.9 ± 0.5	1.8 ± 0.4	4.5 ± 3	1.5 ± 0.5	5.8 ± 3.5	2.2 ± 0.7	4.6 ± 1.9	2.7 ± 0.3	2.9 ± 1.3
Endomorphin 1	4.2 ± 2.5	3.9 ± 3	7.7 ± 3.3	3.8 ± 1.3	11.5 ± 0.1	4.9 ± 2.5	9.9 ± 4	4.6 ± 0.5	5.1 ± 2
Endomorphin 2	34.1 ± 25.4	4.6 ± 1.3	5.6 ± 3	5.4 ± 1.1	19.9 ± 2.2	6.3 ± 2.5	22.5 ± 3.4	9.6 ± 5.2	9.4 ± 5.1
β-Endorphin	3.5 ± 0.1	4.3 ± 2.2	6.6 ± 2.9	7.8 ± 2.7	24.7 ± 8.8	8.2 ± 3.8	16 ± 0.7	10 ± 5.8	8.4 ± 3
Dynorphin A	8.6 ± 0.3	47.4 ± 9.6	8.4 ± 1.9	19.3 ± 11.4	49 ± 38.5	13.8 ± 4	70.5 ± 51.1	52.7 ± 39.3	24.7 ± 22.5
DPDPE	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
U50,488H	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500

Table 4

Clone	K_D (nM)	B_{max} (pmol/mg protein)
rMOR-1	2.8 ± 0.79	2.94 ± 0.45
rMOR-1 no E 4	4.5 ± 1.1	0.39 ± 0.06
rMOR-1A	2.5 ± 0.2	0.81 ± 0.06
rMOR-1C1	4.8 ± 0.6	1.82 ± 0.29
rMOR-1D	4.7 ± 1.2	0.51 ± 0.05

Table 5

(% Maximal Inhibition)	DAMGO	Morphine	B-Endorphin
hMOR-1	84	89	88
hMOR-1 (exons 1-3)	81	83	81
hMOR-1A	90	92	81
hMOR-1B1	63	76	72
hMOR-1B2	99	98	72
hMOR-1B3	80	51	81
hMOR-1B4	71	76	77
hMOR-1B5	91	86	77
hMOR-1Y	100	100	100

Table 6

Ligand	DAMGO			Morphine			β-Endorphin		
	IC_{50}	IC_{50}/K_i	Max Inhib (%)	IC_{50}	IC_{50}/K_i	Max Inhib (%)	IC_{50}	IC_{50}/K_i	Max Inhib (%)
hMOR-1	2 ± 0	1.0	76 ± 4	4 ± 1	0.9	74 ± 6	13 ± 3	0.3	79 ± 2
hMOR-1 (exons 1-3)	3 ± 1	2.0	74 ± 3	5 ± 1	1.7	69 ± 6	16 ± 4	3.7	73 ± 4
hMOR-1A	5 ± 2	2.1	82 ± 1	9 ± 3	2.0	76 ± 1	22 ± 3	3.3	73 ± 2
hMOR-1B1	46 ± 17	38.3	57 ± 2	47 ± 19	19.6	63 ± 2	34 ± 4	4.4	65 ± 4
hMOR-1B2	29 ± 7	5.0	90 ± 3	72 ± 16	6.4	81 ± 1	108 ± 34	4.4	65 ± 5
hMOR-1B3	38 ± 7	21.1	73 ± 8	11 ± 4	3.4	42 ± 1	48 ± 12	5.9	73 ± 9
hMOR-1B4	20 ± 3	8.7	65 ± 3	8 ± 2	1.5	63 ± 2	60 ± 16	3.8	69 ± 5
hMOR-1B5	34 ± 5	16.1	83 ± 1	19 ± 2	4.9	71 ± 1	217 ± 50	21.7	69 ± 8
hMOR-1Y	8 ± 2	3.2	91 ± 2	9 ± 2	2.1	83 ± 3	44 ± 16	5.2	90 ± 2

Characterization of expressed variants. In order to study pharmacological functions of the variants, CHO cell lines stably transfected with individual variant cDNAs whose expression was under control of a human cytomegalovirus (CMV) early promoter, a common promoter used in mammalian expression system, were

5   obtained. As a control, a stable cell line expressing a receptor that only contained exons 1-3 without a fourth exon was included. In saturation studies, [^{3}H] DAMGO revealed high affinity for all the variants with little difference among their K_D values (Table 2). Competition studies with a variety of opioids further demonstrated that all the variants encoded a mu opioid receptor (Table 3). The mu ligands such as

10 DAMGO, morphine and M6G all competed binding with high affinities, while the delta-selective ligand DPDPE and the $kappa_1$-selective opioid, U50,488H were inactive. The control receptor (hMOR-1 (exons 1-3)) showed a high affinity toward [^{3}H] DAMGO in saturation studies and the similar mu-selective binding profile in competition studies, suggesting that the ligand binding was not very dependent on the

15 carboxyl termini encoded by the different fourth exons. It was not surprising because they all had the same N-terminal seven transmembrane structure that comprises binding motifs for mu ligands. However, the variants did display subtle but significant binding pocket differences. For example, endomorphin 2 competed binding to the hMOR-1 over 5- to 7-fold less potently than against hMOR-1A,

20 hMOR-1B1, hMOR-1B3 and hMOR-1 (exons 1-3) ($p < 0.05$), respectively. β-endorphin showed lower affinities toward hMOR-1B2 and hMOR-1B4 as compared to other variants. Interestingly, compared to the other variants, hMOR-1B2 had relative lower affinities for all the mu agonists including DAMGO, morphine, M6G and endomorphin 1, but maintained a high affinity for naloxone. The carboxyl

25 terminal sequence of hMOR-1B2 which encoded by exon 5b was quite unique with four basic residues in a total of nine amino acids. It also contained a potential cAMP- and cGMP-dependent protein kinase phosphorylation site.

Since the structures of all the variants differ from each other only at their carboxyl termini which are all located at intracellular side, there may be differences in

30 their coupling to G protein transduction pathway. Mu opioid receptors are mainly coupled to Gi/Go and associated with inhibition of adenylyl cyclase to reduce the

intracellular level of cAMP, an important second messager system within cells. The effect of several opioids on forskolin-stimulated cAMP accumulation in intact cells expressing the variants was investigated.

5 The results showed striking differences in the effect of opioids on inhibiting cAMP production (Tables 3 and 4). This could be seen in both their potency determined by the IC_{50} values, and efficacy indicated by the percentage of maximal inhibition. DAMGO and morphine, which displayed the limited differences among the variants in binding assays, revealed their IC_{50} values that varied from 3- to 23-fold. β-endorphin IC_{50} values also differed from 3- to 16-fold. The ratio of the

10 IC_{50}/Ki values that takes account for binding affinities of the opioids is another measurement for comparing their potencies. Again, there were marked differences of potencies among the variants. For example, the DAMGO IC_{50}/Ki value for hMOR-1B1 was over 38-fold than that for hMOR-1. Although β-endorphin IC_{50} value varied over 16-fold between hMOR-1 and hMOR-1B5, their IC_{50}/Ki value differed

15 by over 72-fold. Therefore, different carboxyl termini can indeed affect the potency of opioids to inhibit forskolin-stimulated cAMP accumulation in intact cells independent of their binding affinities.

Marked differences in the maximal inhibition (%), an indication of the opioid efficacy, were also observed among the variants (Tables 3 and 4). The ranges of the

20 maximal inhibitions for DAMGO, morphine and β-endorphin were 63 – 100%, 51 – 100% and 72 – 100%, respectively (Table 5). Interestingly, hMOR-1Y had the highest efficacy for all the opioids, whereas the lowest efficacies for the opioids varied among the variants. hMOR-1B1 showed the lowest efficacy (63%) for DAMGO, while hMOR-1B1 and hMOR-1B2 had the lowest efficacy (72%) for

25 morphine. hMOR-1B3 displayed only 51% of efficacy. There was little correlation between their potencies and efficacies. For example, morphine had the same efficacy for hMOR-1B1 and hMOR-1B4, but there was a 13-fold difference in their IC_{50}/Ki values. On the other hand, in binding assays hMOR-1B2 had relatively low affinities toward DAMGO and morphine, but both opioids showed higher efficacies for

30 hMOR-1B2, when compared to those for other variants. It also was interesting to observe that the relative efficacy of the different opioids for an individual variant

varied (Table 5). For example, DAMGO and morphine were more efficacious than β-endorphin against hMOR-1B2, while β-endorphin and DAMGO were more effective than morphine against hMOR-1B3.

There was no association between binding affinity and potency or efficacy among the human variants for a number of drugs, which was similar to results obtained from the mouse MOR-1 variants by using the [^{35}S]GTP(S binding assay (see, e.g., Bolan et al. (2000) Soc.Neurosci. 26, 112), which is another method for assessing receptor signalling. Although different human variants often bind the same ligand equally well, the functional consequences, as indicated by their abilities to inhibit forskolin-stimulated cAMP accumulation, were distinct from each other. One implication of these studies is to potentially develop selective drugs based upon their efficacy and/or potency.

Together with the hMOR-1A, hMOR-1O and hMOR-1X variants previously isolated, there now are a total of nine human splice variants. The extensive alternative splicing first demonstrated in mouse now applies to human. All the nine variants were carboxyl terminal variants resulted from alternative splicing of the fourth exon. The different protein structures at their carboxyl tips had the limited effect on the binding selectivity of the variants, but all dramatically altered signaling thought through their interaction with G protein system. This dissociation of binding affinity and efficacy or potency for the variants, together with their potential region-specific expression, may provide insight to understand different pain thresholds and different opioid responses in human, and to design novel selective or individualized drugs that modulate pain based upon their efficacy/potency.

EXAMPLE 2: Identification and characterization of five new splice variants (rMOR-1C1, rMOR-1C2, rMOR-1D, rMOR-1B2, and rMOR-1E) of the rat *Oprm* gene

Five splice variants (rMOR-1C1, rMOR-1C2, rMOR-1D, rMOR-1B2, and

5   rMOR-1E) of the rat *Oprm* gene from rat brain have been identified. The main

strategy used for isolating the human and rat variants was to search the homologous

variants in rat using the mouse variant sequences either by comparing with public rat

genome databases (NBCI and Ensembl) or by directly using the mouse sequences to

perform RT-PCT. All the rat variants contained the same exons 1, 2 and 3 as their

10   original MOR-1, but had a different fourth exon. Therefore, all of the variants are

carboxyl terminal variants. Although the variants were obtained by using the mouse

sequences and shared some degree of homology at nucleotide level with the mouse

sequences, all the predicted amino acid sequences from the alternative fourth exons

were totally different from any of the mouse sequences identified so far. Northern

15   blot analysis with appropriate exon probes revealed distinct band patterns with

different sizes and intensities.

The variants were expressed in CHO cells and characterized them by opioid

binding, $[^{35}S]GTP\text{-}\gamma\text{-}S$ binding and adenylyl cyclase assays. The binding results

showed that all the variants had higher affinity to mu-specific opioids and lower

20   affinity to kappa or delta drugs, a similar profile revealed by the original MOR-1.

This could be seen in both their potency determined by the IC_{50} values, and efficacy

indicated by the percentage of maximal inhibition. The results from $[^{35}S]GTP\text{-}\gamma\text{-}S$

binding assay of the rat variants revealed marked differences in the ability of the

opioids to stimulate $[^{35}S]GTP\text{-}\gamma\text{-}S$ binding in terms of both their potency determined

25   by the EC_{50} and efficacy indicated as maximal stimulation. The different protein

structures at carboxyl tips among the variants had the limited effect on their binding

selectivity, but they do affect forskolin-stimulated adenylyl cyclase activity,

presumably through their interaction with G proteins, as well as $[^{35}S]GTP\text{-}\gamma\text{-}S$

binding. This dissociation of binding affinity and efficacy or potency for the variants,

30   together with their potential region-specific expression, may provide an important

insight to understand different pain thresholds and different opioid responses in

human and rat, and to design novel selective or individualized drugs that modulate

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pain based upon their efficacy/potency.

Table 7 presents the selectivity of rMOR-1 splice variants in [^{3}H]DAMGO binding assay. Competition studies were performed in [^{3}H]DAMGO (~1 nM) binding assay using at least three concentrations of the indicated ligand with membranes from the same stable transfectant. ANOVA was performed, followed by Tukey's post hoc analysis. Results are the mean ± S.E. of at least three independent determinations.

Table 8 depicts the effects of opioid on [^{35}S]GTPγS binding in the rat MOR-1 splice variants. Basal [^{35}S]GTPγS binding was assessed in membranes from cells stably transfected with the individual variants. The ability multiple concentrations of of the indicated opioid to stimulate [^{35}S]GTPγS binding was determined. The maximal stimulation was defined as the percent increase over basal binding, and the dose of drug needed to elicit 50% of the maximal response, the EC50. Results are the means ± S.E.M. of at least three independent determinations.

Table 7

Ligand	Ki Value (nM)					ANOVA	Tukey	
	rMOR-1	rMOR-1 (no E4)	rMOR-1A	rMOR-1C1	rMOR-1D		rMOR	p
Morphine	5.6 ± 0.8	6.5 ± 0.6	8.0 ± 0.4	7.4 ± 0.3	7.4 ± 0.5	N.S.		
M6G	16.9 ± 2.2	20.5 ± 2.8	25.7 ± 2.1	24.8 ± 2.4	21.0 ± 1.8	N.S.		
DAMGO	3.3 ± 0.6	2.4 ± 0.4	6.0 ± 0.9	4.5 ± 0.9	4.7 ± 1.2	N.S.		
DADLE	4.3 ± 0.5	6.8 ± 1.3	8.1 ± 0.4	8.6 ± 1.0	5.4 ± 0.3	N.S.		
DSLET	30.9 ± 1.9	24.2 ± 1.9	44.5 ± 5.1	45.0 ± 8.7	27.3 ± 7.3	N.S.		
Endomorphin 1	4.1 ± 0.7	4.4 ± 0.1	6.5 ± 0.3	3.9 ± 0.1	3.9 ± 0.4	< 0.05	1 vs 1A	< 0.05
							1A vs 1C1	< 0.05
							1A vs 1C2	< 0.05
Endomorphin 2	8.0 ± 2.0	9.1 ± 0.7	11.5 ± 0.6	10.1 ± 0.6	7.5 ± 0.4	N.S.		
β-Endorphin	3.7 ± 0.4	10.0 ± 0.8	10.5 ± 0.6	8.8 ± 0.5	8.5 ± 0.6	< 0.0005	1 vs 1(no E4)	< 0.001
							1 vs 1A	< 0.001
							1 vs 1C1	< 0.01
							1 vs 1C2	< 0.01
Dynorphin A	12.4 ± 3.0	14.0 ± 1.4	22.8 ± 1.6	12.5 ± 2.3	10.8 ± 1.7	< 0.05	1A vs 1C2	< 0.05
Naloxone	3.2 ± 0.4	4.1 ± 0.4	5.0 ± 0.9	5.4 ± 0.9	2.6 ± 0.8	N.S.		
U50,488H	> 500	> 500	> 500	> 500	> 500			
DPDPE	> 500	> 500	> 500	> 500	> 500			

Table 8

Ligand	rMOR-1			rMOR-1 (no Exon 4)			rMOR-1A			rMOR-1C1			rMOR-1D		
	$EC50$ (nM)	$EC50/Ki$	Max Stim (%)	$EC50$ (nM)	$EC50/Ki$	Max Stim (%)	$EC50$ (nM)	$EC50/Ki$	Max Stim (%)	$EC50$ (nM)	$EC50/Ki$	Max Stim (%)	$EC50$ (nM)	$EC50/Ki$	Max Stim (%)
DAMGO	12±3	3.6	233±44	86±16	35.8	196±37	13±5	2.2	176±24	74±22	16.4	233±45	125±26	26.6	113±22
DADLE	24±5	5.6	298±26	401±38	59.0	225±24	63±23	7.8	175±8	128±15	14.8	379±62	300±29	55.6	204±23
DSLET	76±17	2.5	251±5	690±179	28.5	226±15	127±45	2.9	206±23	253±75	5.6	312±19	540±129	19.7	187±19
Endomorphine1	14±4	3.4	320±23	114±11	25.9	189±16	15±3	2.3	205±34	54±8	13.8	377±40	100±26	25.6	145±29
β-Endorphin	4±2	1.1	246±52	90±20	9.0	240±16	13±5	1.2	177±28	48±4	5.5	361±41	91±14	10.7	165±42

All references cited herein, are hereby incorporated herein. Although the foregoing invention has been described in some detail, by way of illustration and example for the purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practice. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims and/or claims.

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